Simultaneous quantification of steroid hormones and endocannabinoids (ECs) in human hair using an automated supported liquid extraction (SLE) and LC-MS/MS – Insights into EC baseline values and correlation to steroid concentrations

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Simultaneous quantification of steroid hormones and endocannabinoids (ECs) in human hair using an automated supported liquid extraction (SLE) and LC-MS/MS – Insights into EC baseline values and correlation to steroid concentrations

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ABSTRACT

Endogenous steroid hormones and endocannabinoids (ECs) are important regulators in the stress response of the human body. For the measurement of chronic stress, hair analysis has been established as method of choice for long-term and retrospective determination of endogenous stress markers. A sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of five steroid hormones (cortisone, cortisol, androstenedione, testosterone, progesterone) and four endocannabinoids (anandamide, palmitoylethanolamide, 2-arachidonylglycerol, oleoylethanolamide) in hair was developed and validated. The hair samples were extracted with methanol and cleaned up with a fully automated supported liquid extraction (SLE) before analysis. Special attention was paid to the difficulties accompanying the quantification of endogenous analytes in hair.

Five different strategies for endogenous compound quantification in hair (surrogate analyte, standard addition, background correction, stripped matrix and solvent calibration) were tested and compared. As a result, the approach of the surrogate analyte was used for the quantification of steroid hormones whereas background correction was used for endocannabinoids. The measurement of 58 samples from healthy young adults allowed insights into endocannabinoid ranges in hair and the correlation to steroid hormones. No significant differences in steroid and EC concentration levels of male and female in hair were found, except for testosterone ($p < 0.001$) and androstenedione ($p < 0.0001$). Cortisol to cortisone and testosterone to androstenedione concentrations were significantly and positively correlated. There were significant intercorrelations between endocannabinoids.

1. Introduction

Steroid hormones and endocannabinoids (ECs) play an important role in the physiology and behavioral expression of the human stress response. The hormonal response to stress is activated along the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is regulated by cortisol and related steroids [1,2]. Over the short-term, activation of the HPA axis is helpful to cope with stressful events; over the long-term, a dysregulated HPA axis is associated with a wide range of negative effects on the body (e.g. depression, decreased immune system, hypertension etc.) [3]. More and more studies indicate that the endocannabinoid system is an additional regulator of stress and the HPA axis [1]. The endocannabinoid system consists of receptors, enzymes and several endogenous ligands (endocannabinoids) binding to the cannabinoid

Abbreviations: 2-AG, 2-arachidonylglycerol; AEA, anandamide; APCI, atmospheric pressure chemical ionization; ECs, endocannabinoids; ESI, electrospray ionization; GC-MS, gas chromatography-mass spectrometry; HPA, hypothalamic-pituitary-adrenal; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring mode; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; PTSD, post-traumatic stress disorder; RSD, relative standard deviation; SLE, supported liquid extraction.

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(CB) receptors, i.e. CB_1 and CB_2 [4]. The most investigated endocannabinoids are *N*-arachidonylethanolamine (anandamide, AEA) and 2-arachidonylglycerol (2-AG) which are known to be involved in affect, pain and stress regulation [5]. Other lipids that are associated with the EC system are palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) which do not directly bind to the CB receptors but might modify AEA and 2-AG [6,7]. Most explanatory models are suggesting that cortisol and endocannabinoids are interlinked and that glucocorticoids induce fast feedback inhibition of the HPA axis [8].

Cortisol concentrations are highly fluctuating in plasma, saliva or urine underlying the circadian rhythm with usually highest concentrations in the morning. The cortisol concentration in these classical matrices is depending on day time and other specific circumstances like food intake or alcohol consumption [9]. Keratinized matrices, such as hair, can reflect cumulative steroid levels over extended time periods (up to several months) [9]. In recent years, hair analysis has become an important tool for retrospective stress monitoring [3,10,11]. A number of studies have been performed in this field that investigated hair cortisol across a range of stress-related conditions in humans [12]. Increased hair cortisol has been shown to be associated with stressful conditions such as chronic pain [13], pregnancy [14], athletics [15] or alcoholism [16]. Research has also started to focus on the measurement of endocannabinoids as a biological regulator for stress [1]. It is known that also endocannabinoids like 2-AG underlie significant circadian changes and the concentration is changing during the day [17]. Because of the large number of physiological factors that can contribute to the changes of EC concentrations in body fluids, a reliable stress monitoring in blood, urine and saliva is challenging. Therefore, more and more studies use hair EC measurements for stress monitoring similar to hair cortisol. First studies revealed a correlation between endocannabinoids in hair and post-traumatic stress disorder symptoms (PTSD) [18], borderline personality disorder [19], childhood mistreatment [20], burnout, depression, anxiety symptoms [21] and obesity [22].

The analysis of endocannabinoids is an analytical challenge due to their low levels in biological matrices and their chemical similarity. First efforts for quantifying endocannabinoids were performed by gas chromatography-mass spectrometry (GC-MS) [23]. Nowadays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is mainly used for the quantification of steroid hormones and ECs in biological matrices and the most common ionization techniques for the determination of endocannabinoids are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) [7,21,22,24]. Even though an increasing number of LC-MS/MS methods was developed to measure ECs in body fluids, poor agreement on sample collection, storage and preparation exists [25]. Also for the measurement of ECs in hair only a few publications exist to date [18–22]. For ECs, various pitfalls in the pre-analytics and analytics are known. The extraction should minimize contamination and isomerization. It was reported that several solvents can raise contamination of endocannabinoids and thus can be a challenge for the reliable detection of ECs [25]. Moreover, it is known that 2-AG undergoes rapid isomerization to 1-AG whereas complete avoidance seems to be impossible [7]. It is therefore important to extract the biological matrix in neutral solutions. The measurement of steroid hormones in hair, especially cortisol, is widely used and baseline values are defined whereas concentrations ranges for ECs are not established yet [26].

Endogenous compounds like steroids and ECs are more challenging to quantify and to validate than xenobiotic compounds. The FDA (Food and Drug Administration) states in its recommendation for method validation that the use of authentic matrix to spike with the analytes of interest is the best option [27]. For endogenous compounds, this is challenging, as there is commonly no authentic analyte-free matrix available. In some cases, analyte concentrations in the hair matrix are sufficiently low but this is rather rare. Several approaches have been described to overcome this problem like the use of standard addition, the use of surrogate matrix, the method of background correction or the use of surrogate analytes [28–30]. One possibility to obtain analyte-free matrix is to use a stripped matrix where endogenous compounds are actively removed from the hair or to use hair tips from long hair [28,29,31]. Hair tips can be used if the concentration of endogenous compounds is sufficiently low because of a general wash out effect [31]. Furthermore, the approach of surrogate analytes was applied successfully for the quantification of several steroid hormones in hair [32,33].

Overall, this study aimed to develop and validate an LC-MS/MS method for the simultaneous quantification of steroid hormones and endocannabinoids in hair. Special attention was paid to find a suitable quantification model for simultaneous determination of endogenous steroids and endocannabinoids in hair. Moreover, we applied the method to authentic hair samples to gain insights into endocannabinoid ranges in the hair matrix of healthy volunteers and to investigate the correlations to steroid hormones.

2. Material and methods

2.1. Chemical reagents

Oleoylethanolamide, palmitoylethanolamide and deuterated internal standards (cortisone-D_7, progesterone-D_9) were purchased from Sigma Aldrich (Buchs, Switzerland). Anandamide, 2-arachidonylglycerol and the deuterated internal standard (AEA-D_{11}) were purchased from Cayman Chemicals (Ann Arbor, USA). $^{13}C_3$-cortisol and $^{13}C_3$-cortisone were purchased from Isoscience (Ambler, USA) and $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone and $^{13}C_3$-progesterone from Sigma Aldrich (Buchs, Switzerland). Water and methanol were of LC–MS grade (Chromasolv®) and purchased from Sigma-Aldrich (Buchs SG, Switzerland). Acetone, ethyl acetate and ammonium fluoride were purchased from Merck (Darmstadt, Germany). Reconstitution solution consisted of 0.2 mM NH_4F in water/methanol 97/3 v/v. Isolute® SLE + columns were purchased from Biotage® (Uppsala, Sweden). All chemicals were of highest analytical grade.

2.2. Preparation of standard stock solutions

Final concentrations of 1 ng/μL of standards of each analyte were prepared in MeOH for the steroid hormones and in acetonitrile for the endocannabinoids. The internal standard mixture (cortisone-D_7, progesterone-D_9, AEA-D_{11}) was prepared in acetonitrile at a final concentration of 40 pg/μL for progesterone-D_9 and AEA-D_{11} and 80 pg/μL for cortisone-D_7. For calibration and validation experiments, stock solutions were prepared in different concentrations: 2, 20 and 200 pg/μL for $^{13}C_3$-cortisol, $^{13}C_3$-cortisone, $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone and $^{13}C_3$-progesterone, 0.2, 2 and 20 pg/μL for AEA, 2, 20 and 200 pg/μL for 2-AG and 200, 2.000 and 10.000 pg/μL for PEA and OEA. All stock solutions were stored at −20 °C until use.

2.3. Sample collection and ethical aspects

For validation experiments, several different hair pools from healthy volunteers were prepared by snipping the hair and homogenizing it. For baseline values, authentic hair samples were measured from students that were recruited in the framework of a collaboration study (JurSTRESS, University of Regensburg, Germany). The present study was performed in full conformance with Swiss and German laws, particularly those pertaining to use of human materials in research. A statement of the Cantonal Ethics Board of the Canton of Zurich (document BASEC-Nr. Req-2017-00946) and the Ethics Board of the Faculty of Medicine of the University Regensburg, Germany (Nr. 14-101-0064) was obtained. Hair samples were collected from the posterior vertex region and cut as close to the scalp as possible. The 3 cm hair segment proximal to the scalp was analyzed. This segment represents the last three months prior to the sampling, based on an average hair growth rate of 1 cm/month.

2.4. Sample preparation and extraction

Hair samples were segmented and washed for 3 min with 15 mL of deionized water, followed by washing for 2 min with 10 mL acetone. The Sarstedt tubes were shaken by hand during the washing process. The washing solutions were decanted and disposed. The samples were dried overnight at room temperature. After washing, the hair segments were cut into snippets. For extraction 20 mg hair sample was weighed in a 2 mL Eppendorf tube and 50 μL internal standard (cortisone-D_7, progesterone-D_9, AEA-D_{11}) and 1 mL methanol were added. The samples were briefly shaken and placed in a sonication bath (35 kHz, 600 W) for 4 h at 55 °C for extraction. After centrifugation for 5 min at 9000 g, the methanolic extract was transferred into the column rack (24 × 6 mL) from Biotage® Extrahera (Biotage, Uppsala, Sweden). Sample extracts were automatically loaded onto Isolute SLE + columns and allowed to absorb for 5 min. Analytes were then eluted two times with 2.5 mL ethyl acetate with a wait time of 5 min in-between. The extracts were dried in a Turbovap® (Biotage, Uppsala, Sweden) and resuspended in 60 μL MeOH and 140 μL reconstitution solution.

2.5. LC-MS/MS analysis

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) device was used for the analysis of the sample extracts. Analytes were separated with a Prominence UFLC system (Shimadzu, Kyoto, Japan) by injecting 10 μL of the samples onto a Phenomenex® Kinetex® XB-C_{18} (2.6 μm, 50 × 2.10 mm) column. The mobile phase consisted of 0.2 mM NH_4F in water/methanol 97/3 v/v (A) and 0.2 mM NH_4F in water/methanol 3/97 v/v (B). The flow rate was 0.40 mL/min and the temperature of the column oven was set to 40 °C. Separation was achieved by the following gradient: 0–40% B for 0–0.1 min, isocratic 40% from 0.1 to 1 min, 40–70% B from 1 to 5 min, isocratic 70% for 5–6 min, 70–80% from 6 to 7 min, isocratic 80% for 7–11 min, 80–90% from 11 to 14 min, isocratic 99% B from 14.1 to 17 min, 99–40% B from 17 to 19 min followed by an equilibration step of 1 min. Detection was performed with a QTRAP® 6500+ linear ion trap quadrupole mass spectrometer (Sciex®, Darmstadt, Germany) equipped with an electrospray ionization (ESI) source. Analytes were measured in positive ESI mode. Multiple reaction monitoring mode (MRM) with an ion spray voltage of 4500 V was used for quantification (see Table 1). The method parameters are listed in Table 1. The curtain gas was fixed at 20 psi, the collision gas was set to high, the ion source gas 1 at 70 psi and ion source gas 2 at 50 psi. The source temperature was set to 600 °C. Analyst® software (version 1.6.3, Sciex®, Darmstadt, Germany) was used for instrument control and data analysis.

2.6. Quantification model

The hair matrix naturally contains individual endogenous concentrations of steroids and ECs. Therefore, it is challenging to find a true "blank" hair matrix for validation and calibration. Five different strategies were tested to circumvent the lack of blank matrix for the quantification of the above-mentioned compounds. For the quantification of steroid hormones in hair, the surrogate analyte approach in adaption to our previous work was used [32–34]. $^{13}C_3$-cortisol, $^{13}C_3$-cortisone, $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone, and $^{13}C_3$-progesterone were used for calibration of steroids. The end concentrations for calibration were 0, 1, 5, 10, 50, 100, 200 pg/mg for $^{13}C_3$-cortisol $^{13}C_3$-cortisone, $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone and $^{13}C_3$-progesterone, respectively.

For ECs, $^{13}C_3$-labeled analogues were not commercially available which is why alternative approaches were tested, namely: standard addition, background correction, stripped matrix and solvent calibration.

For standard addition, four different hair pools were divided into equal aliquots and spiked with EC standards with the following end

Table 1
MRM transitions and MS parameters for all analytes and the internal standards. For each substance, the most sensitive transition was used for quantitation (quantifier) and the second sensitive was used for confirmation (qualifier). RT = retention time, DP = declustering potential, EP = entrance potential, CE = collision energy, CXP = cell exit potential.

Analyte	Q1 mass [Da]	Q3 mass [Da]	RT [min]	DP [V]	EP [V]	CE [V]	CXP [V]
Cortisone 1	361.0	163.0	2.87	145	10	46	11
Cortisone 2	361.0	121.1	2.87	145	10	46	11
Cortison-D_7	368.0	169.0	2.87	145	10	46	11
Cortisone$^{13}C_3$ 1	364.0	166.0	2.87	76	10	31	8
Cortisone$^{13}C_3$ 2	364.0	124.1	2.87	76	10	37	6
Cortisol 1	363.0	121.0	3.20	145	10	46	11
Cortisol 2	363.0	90.9	3.20	6	10	31	10
Cortisol$^{13}C_3$ 1	366.0	124.0	3.20	56	10	29	18
Cortisol$^{13}C_3$ 2	366.0	330.1	3.20	56	10	29	18
Androstenedione 1	287.1	97.0	4.75	145	10	46	11
Androstenedione 2	287.1	109.2	4.75	145	10	46	11
Androstenedione$^{13}C_3$ 1	290.1	100.2	4.75	61	10	31	16
Androstenedione$^{13}C_3$ 2	290.1	112.1	4.75	145	10	46	11
Testosterone 1	289.1	97.0	5.14	145	10	46	11
Testosterone 2	289.1	109.0	5.14	145	10	46	11
Testosterone$^{13}C_3$ 1	292.1	100.0	5.14	91	10	27	14
Testosterone$^{13}C_3$ 2	292.1	111.9	5.14	91	10	31	16
Progesterone 1	315.1	97.0	6.30	145	10	46	11
Progesterone 2	315.1	108.9	6.30	145	10	46	11
Progesterone-D_9	324.1	100.0	6.30	145	10	46	11
Progesterone$^{13}C_3$ 1	318.1	100.0	6.30	26	10	29	16
Progesterone$^{13}C_3$ 2	318.1	112.1	6.30	26	10	31	12
AEA 1	348.2	287.1	9.75	26	10	13	10
AEA 2	348.2	61.9	9.75	26	10	13	10
AEA-D_{11}	359.3	61.9	9.75	1	10	13	8
PEA 1	300.1	62.1	10.16	36	10	13	16
PEA 2	300.1	283.3	10.16	36	10	15	14
2-AG/1-AG 1	379.0	287.1	10.20/10.50	21	10	17	14
2-AG/1-AG 2	379.0	269.1	10.20/10.50	21	10	13	12
OEA 1	326.2	62.1	10.81	150	10	13	6
OEA 2	326.2	69.1	10.81	46	10	13	6

concentrations: 0, 0.5, 1, 2, 5, 10, 20 pg/mg for AEA, 0, 5, 10, 50, 100, 500, 1.000 pg/mg for 2-AG, 0, 500, 1.000, 2.000, 4.000, 10.000, 15.000 pg/mg for PEA and OEA (see Table S1 in the supplementary). A calibration curve was determined and the concentration of each hair pool was then calculated from the negative x-intercept of the linear regression line.

For background correction, 20 mg of one hair pool for each calibrator was spiked with six different concentrations (Table S1) and a standard addition was performed to determine the endogenous concentration of ECs. The calibrator concentrations were corrected by adding the endogenous concentrations of the ECs to the spiked concentration. This resulted in a corrected calibration curve, which was then used for further EC quantification in other hair samples.

To generate analyte-free matrix (stripped matrix), 500 mg hair tips from one individual were extracted in 20 mL MeOH consecutively for seven days. The MeOH was exchanged every day. After drying, 20 mg of the stripped hair pool was spiked with different EC concentrations (Table S1) to obtain a calibration curve.

For solvent calibration, different concentrations of standards (Table S2) were added to matrix-free solvent (MeOH) and mixed with reconstitution solution. The mix was directly injected, i.e. no hair matrix was added to the solutions.

2.7. Method validation

The validation was performed in accordance to the guidelines of the GTFCh appendix C for hair [35]. The following parameters were evaluated: response factor (for the surrogate analytes), linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, matrix effect, recovery and robustness.

For steroid hormones, validation parameters were tested using $^{13}C_3$-labeled surrogate analytes as described before [32–34,36]. Firstly, the response factor was calculated by the ratio of the responses found for surrogate and authentic analyte. If the response factor was not 1 it was incorporated into the regression line of the calibration curve in order to get the correct result for the quantification of the endogenous analyte.

For endocannabinoids, validation parameters were determined by background correction as described above. For the calibration curve, the endogenous amount of EC concentrations was mathematically added to the spiked calibrator concentration (in pg/mg) resulting in a corrected calibration curve. For accuracy and precision, the quality control (QC) end concentrations were calculated by subtracting the endogenous hair pool concentration from the calculated concentration. For matrix effect and recovery, the peak area of the endogenous background concentration in the hair pool was subtracted from the peak area of the measured concentration.

In general, validation parameters for steroid hormones and ECs were calculated as follows: For linearity, six calibrators with increasing concentrations and an unspiked sample (zero sample) were prepared. The regression lines were calculated using a simple linear model with 1/x weighting for steroid hormones and no weighting for ECs. LOD and LOQ were calculated and determined applying signal-to-noise ratios of 3:1 and 10:1, respectively. For accuracy and precision, duplicates of a hair pool with different end concentrations according to substance classes were prepared (Table S4 in the supplementary). The measurements were carried out on six consecutive days. The bias as well as the intra- and inter-day precision was calculated for each analyte. For matrix effect and recovery, five replicates from different individuals at two different concentration levels were analyzed (Table S5). For the matrix effect, the ratio of peak areas of spiked hair (A) to spiked solvent (B) at the same concentration was compared. (Matrix effect = (A/B) x 100). For recovery, the ratio of peak area of spiked matrix before (C) and after (D) extraction was compared. (Recovery = (C/D) x 100). For robustness, 12 replicates of an authentic hair pool were analyzed and the mean value of detectable analytes plus the relative standard deviation was determined.

2.8. Statistical analysis

Statistical analysis was conducted with GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Shapiro Wilk normality test revealed that the data were not normally distributed. Paired samples were compared using Wilcoxon signed rank test and unpaired samples were compared using Mann-Whitney test. Correlation coefficients were calculated using Spearman correlation. *P*-values > 0.05 were considered as not statistically significant (ns); $p < 0.05$ (*) as significant; $p < 0.01$ (**) as very significant; $p < 0.001$ (***) and $p < 0.0001$ (****) as extremely significant.

3. Results and discussion

3.1. LC-MS/MS method

In hair, first methods have been established for the simultaneous quantification of cortisol, cortisone, AEA and 2-AG [22,24]. With this newly developed LC-MS/MS method, several improvements have been achieved. To the best of our knowledge, it is the first method for the simultaneous quantification of five steroid hormones and four endocannabinoids in hair. Our method enables the combined determination of these endogenous compounds in one LC-MS/MS run.

Cortisone, cortisol, androstenedione, testosterone, progesterone, anandamide, 2-arachidonylglycerol, oleoylethanolamide and palmitoylethanolamide in hair could be detected (see Fig. 1). All peaks were well resolved and appeared as single peak. 2-AG and its isomer 1-AG were separated. In contrast to plasma, where it was shown that 1-AG and 2-AG levels can change over the duration of storage [37], the hair matrix is a solid fiber matrix where analyte concentrations are more stable. Most methods describe the determination of 2-AG by integrating both peaks [17,38]. Thus, the concentration of 2-AG in this method was always calculated as a sum of both peaks.

Positive electrospray ionization (ESI) as well as atmospheric pressure chemical ionization (APCI) can be used for the detection of 2-AG and AEA. The current method was based on ESI positive mode. It was demonstrated that the ESI-based method could detect very low EC levels as well as steroid levels in the hair matrix. In contrast to a previously published paper [22], no improvement in sensitivity could be shown when using APCI mode for our method (data not shown). It is known that the ionization source is specific for every manufacturer, thus can lead to different ion source reactions resulting in varying sensitivity.

The extraction of endocannabinoids is a crucial step in the sample treatment. The herein established extraction was using methanol followed by an automated sample clean-up. Supported liquid extraction (SLE) is analogous to liquid-liquid extraction (LLE) but uses an inert support. The sample is absorbed on the inert support material and an organic solvent flows through the support. The final extract is dried afterwards and can then be used for further analysis. It was shown, that SLE clean-up allowed an efficient recovery of all analytes and decreased background noises. The automated clean-up on the Biotage® Extrahera allowed fast and efficient sample through-put. In comparison to previously published work about quantification of endocannabinoids in hair [19–22,24], the implemented methanolic extraction was much shorter (4 h). This allows a minimum exposure to plastic and glass surfaces which is a known source of contamination in endocannabinoid analysis [7].

3.2. Choice of quantification model

Even though several approaches exist for the quantification of endogenous compounds, it remains a challenge to find the adequate approach for the analytes of interest. Especially in hair analysis, it is difficult to find blank matrix for quantification of endogenous compounds and no suitable surrogate matrix is commercially available. For steroid hormones, the approach of using $^{13}C_3$-labeled analogues was

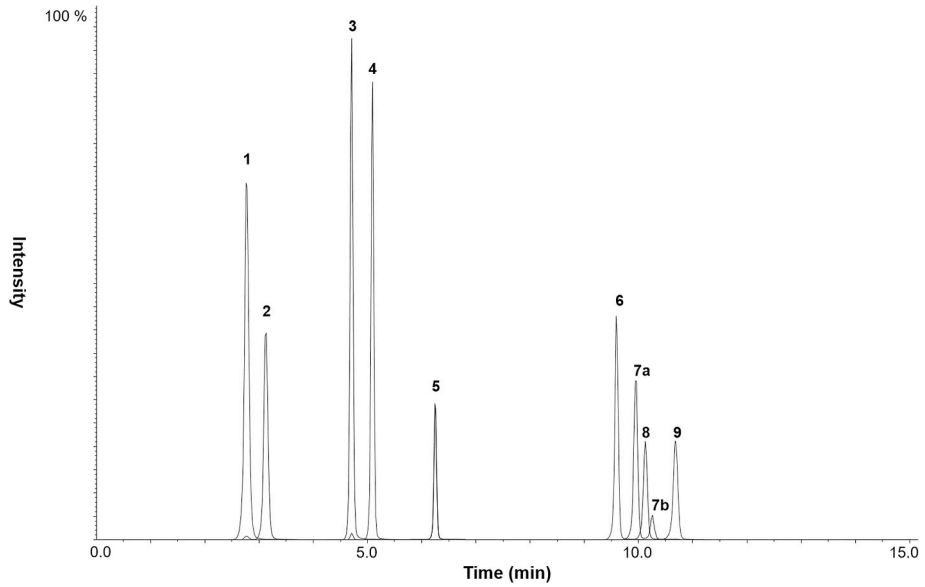

Fig. 1. Chromatogram of the analytes measured by LC-MS/MS in MRM positive mode. 1 = cortisone, 2 = cortisol, 3 = androstenedione, 4 = testosterone, 5 = progesterone, 6 = AEA, 7a = 2-AG/1-AG, 8 = PEA, 7b = 2-AG/1-AG, 9 = OEA.

successfully implemented in hair and nails in the recent years [32–34, 36]. The use of the surrogate analytes allowed a reliable quantification of several steroid hormones especially in low concentration ranges. As this strategy has been proven to be elegant and reliable, it was also used in this study for the quantification of steroids. However, for ECs, no $^{13}C_3$-labeled analogues were commercially available. Thus, different approaches for the quantification of ECs were tested, because of the lack of true blank matrix for endocannabinoids in hair. In general, four different hair pools were tested and quantified with different calibration models. Firstly, standard addition was used to determine the endogenous concentrations of endocannabinoids in each of the four hair pools. In forensic toxicology, standard addition can be used for the quantification of xenobiotics in matrices like blood and urine. In hair analysis, the use of standard addition is often challenging and cannot be performed because of the small sample amount available. Thus, other approaches (background correction, stripped matrix and solvent calibration) were tested and compared to the concentrations obtained by standard addition. The goal was to find an adequate calibration curve that reliably quantifies the concentrations of endocannabinoids in each hair sample without the need to generate a standard addition for each sample. The difference of the concentrations compared to standard addition was calculated in percent (Table 2). The background correction showed reliable results for all four endocannabinoids in a range of -6.5% to 13.2% difference. The background correction has the advantage that the calibration curve is generated in authentic matrix and can then be used for the quantification of different samples. It still has to be taken into account that the hair pool used for the calibration curve can still contain low levels of endocannabinoids. If different batches of a pool are used, different background levels have to be corrected which can lead to deviations in concentrations. Thus, it can become difficult to find an optimal matrix for the calibration curve. The stripped matrix showed good results for some of the endocannabinoids (2-AG, AEA) but for PEA and OEA it showed high differences in the determined concentration (45.1%/-45.0%) compared to the standard addition. That can be explained by high endogenous amounts of PEA and OEA in authentic hair. Therefore, it was not possible to remove these substances fully out of the hair matrix. Furthermore, stripping the hair matrix can change the hair structure and lead to different matrix effects and extraction recovery than in the original matrix. Another possibility of a surrogate matrix is the use of neat solvent for the preparation of the calibration curve. If solvent is used as a matrix, it has to be shown that the extraction recovery and matrix effects are similar to the original matrix. The differences compared to the standard addition were high for all endocannabinoids when using solvent calibration (−95.7% to 99.9%), indicating that solvent calibration is not suitable for quantification of ECs in hair. In our opinion, this can be attributed to the fact that hair is a solid matrix, which differs structurally from liquid matrices. For other applications, it was shown that solvent calibration was an adequate method for the quantification of xenobiotics in various body tissues and body fluids [39].

In conclusion, the comparison of these different methods clarified that it is important to use authentic hair matrix for the quantification of endogenous compounds and that surrogate matrices (stripped matrix, solvent) were not an adequate approach for the quantification of endocannabinoids in hair. For validation and quantification of authentic samples, the approach of background correction was used.

Table 2
Comparison of the concentration difference of endocannabinoids compared to standard addition in percent. The concentrations were firstly calculated by standard addition and then compared to concentrations determined with background correction, a calibration in stripped matrix and solvent calibration. Results are shown as mean difference and standard deviation.

Compound	Background correction (%)	Stripped matrix (%)	Solvent calibration (%)
AEA	−4.3 ± 19.3	1.6 ± 27.7	−95.7 ± 3.4
PEA	12.6 ± 4.8	45.1 ± 14.3	99.9 ± 32.7
2-AG/1-AG	−6.5 ± 4.3	−3.2 ± 4.1	−39.4 ± 4.6
OEA	13.2 ± 10.1	−45.0 ± 13.3	97.8 ± 35.6

3.3. Validation

The validation parameters were determined with help of surrogate analytes for the steroid hormones and with background correction for the ECs and are provided in the supplementary.

LOD, LOQ and correlation coefficient are displayed in Table S3 in the supplementary. Linearity was acceptable for all analytes with a correlation coefficient of more than 0.99. For the surrogate analytes, the response factor was determined and integrated into the regression line (Table S3). The LODs for AEA (0.3 pg/mg) and 2-AG (1 pg/mg) were in similar range compared to previous published methods [21,22]. The

LOD for the steroid hormones was 0.1 pg/mg or below. For OEA and PEA, the LOD could not be determined because of the high endogenous concentration in hair.

Accuracy was in a range of −15.9% up to 14.2% for all analytes (Table S4 in the supplementary). Intra-day precision (RSD_r) was 20% or less for all analytes. All the inter-day values (RSD_T) were less than 23%. The variability was generally higher for samples at low concentration. An explanation for this could be that the amount of spiked analyte is relatively low as compared to the endogenous level resulting in a higher variability.

Recovery was over 80% for all analytes, which is acceptable considering the complexity of the matrix. For the matrix effect, the difference of peak areas of spiked hair extract compared to spiked solvent should be similar. For some of the analytes ion suppression or ion enhancement occurred, thus the delta area was either lower or higher (Table S5 in the supplementary).

To demonstrate the robustness of the method, an authentic hair pool was measured 12 times over a time period of one week. The mean concentration was 29.8 pg/mg (RSD, 9.8%) for cortisone, 6.2 pg/mg (RSD, 11.1%) for cortisol, 0.9 pg/mg (RSD, 15.9%) for androstenedione, 0.9 pg/mg (RSD, 20.3%) for testosterone, 1.2 pg/mg (RSD, 14.0%) for progesterone, 1283 pg/mg (RSD, 10.4%) for PEA, 22.2 pg/mg (RSD, 15.2%) for 2-AG, 740 pg/mg (RSD, 9.2%) for OEA. AEA was not detectable in the authentic hair pool. These data prove the robustness and precision of the analytical method.

In conclusion, the validation proved that the combined strategy of surrogate analyte and background correction could be used for the determination of low endogenous levels of steroid hormones and ECs in hair.

3.4. Analysis of authentic hair samples

LC-MS/MS analysis was performed on 58 hair samples (20 males/38 females, mean age: 23, age range: 21–31) from healthy young adults. The mean concentrations determined in the proximal segment of 3 cm length were 12.2 pg/mg for cortisone, 3.6 pg/mg for cortisol, 1.6 pg/mg for androstenedione, 0.7 pg/mg for testosterone, 2.9 pg/mg for progesterone, 1.9 pg/mg for AEA, 6046 pg/mg for PEA, 113 pg/mg for 2-AG/1-AG and 7006 pg/mg for OEA (Table 3). These ranges were in accordance with previously reported values of steroid hormones and endocannabinoids in hair [20,21,24].

Males showed significantly higher testosterone ($p < 0.001$) and androstenedione ($p < 0.0001$) concentrations than females. For the other steroids (cortisone, cortisol, progesterone) and ECs, no significant differences in concentration levels in hair of male and female were found. Previous literature described gender-dependent concentration ranges for steroid hormones and ECs [11,22,26,40]. Higher testosterone levels in males are probably due to the production of this hormone mainly in the testes in males [41].

From our data, correlations for steroid hormones and endocannabinoids were calculated. AEA showed a significant, positive correlation with 2-AG ($r = 0.41$, $p < 0.01$), OEA ($r = 0.36$, $p < 0.01$) and PEA ($r = 0.31$, $p < 0.05$). 2-AG was also significantly and positively correlated with OEA ($r = 0.46$, $p < 0.001$) and PEA ($r = 0.34$, $p < 0.01$). OEA and PEA had a strong positive correlation ($r = 0.92$, $p < 0.0001$). A positive correlation was found between cortisol and cortisone ($r = 0.35$, $p < 0.01$) and testosterone and androstenedione ($r = 0.75$, $p < 0.0001$). The correlation between cortisol and cortisone concentrations in hair has been described before [42]. Androstenedione is converted to testosterone via 17β-hydroxysteroid dehydrogenase and thus also reflects an indirect measurement of the enzyme activity [43]. The results showed that increased AEA concentrations are associated with higher 2-AG levels, which is in accordance with previous results [22]. No significant correlation was found between endocannabinoids and steroids. There are literature examples that describe a correlation of endocannabinoids and steroids in plasma and serum. A study by Hill et al. examined the influence of EC levels in individuals with PTSD [44]. In this study, different correlation analyses were performed to assess plasma associations among ECs as well as the relationship to cortisol. No significant relationship of 2-AG to AEA, PEA and OEA was found whereas AEA, PEA and OEA did show intercorrelations with each other. The results were similar in the control group as well in the PTSD group. Furthermore, they could show that cortisol correlated with OEA, PEA and AEA, which they concluded indicates a positive association of ECs with circulating cortisol. However, no correlation was found for cortisol and 2-AG [44]. Another study found no correlation between AEA levels and progesterone in pre- and postmenopausal women [45]. In saliva, cortisol and 2-AG levels were negatively correlated [46].

For the hair matrix, literature provides examples of both the presence and absence of correlations between steroids and ECs [19,21,22,24]. The newest literature by Gao et al. found positive intercorrelations between OEA, PEA and SEA [21]. This is in accordance with our results. Mwanza et al. found a positive correlation between 2-AG and AEA as well as a positive correlation of 2-AG with cortisol and cortisone [22], whereas Krumbholz et al. described a negative correlation between ECs and glucocorticoids [24]. Wingenfeld et al. reported no significant correlation between 1-AG/2-AG as well as no correlation between cortisol and AEA and cortisol [19]. These studies show that contradicting literature exists and depending on the matrix as well as the study cohort, different or no correlations are found between steroids and endocannabinoids. The discrepancy of the results could be attributed to the different study conditions that were investigated in these studies and shows the complexity of the stress system and the need for further research in this field.

Since changes in endocannabinoid levels are known to be of limited duration [47], it is also unclear if small changes in the EC concentrations following a stress response can be detected in hair. The window of detection for the hair matrix is several weeks up to several month, thus reflecting long-term cumulative effects. Temporary fluctuations that are quickly regulated in blood might not be detected using hair as matrix. Furthermore, substances can be incorporated into the hair matrix via different pathways, e.g. through blood, sebum or sweat. Therefore, blood correlations might also be reflected in the hair matrix but sebum and sweat effects could influence the levels of steroids and endocannabinoids, making interpretation challenging. More studies on larger cohorts could help to understand the interactions between endocannabinoids, glucocorticoids and the HPA axis in more detail.

This study was a first attempt to investigate endocannabinoid levels of healthy volunteers in the hair matrix. For a better comparison of endocannabinoid levels, establishment of normative baseline concentrations for ECs could be very useful. Furthermore, studies on the influence of hair color, washing behavior and age should be carried out.

Table 3
Mean, median, 1st, and 3rd quartile concentrations (pg/mg) of steroids and endocannabinoids in 3 cm proximal hair segments of 58 individuals.

Compound	Mean concentration (pg/mg)	Median concentration (pg/mg)	1st and 3rd quartile (pg/mg)
Cortisone	12.2	11	7.9; 13.9
Cortisol	3.6	2.1	1.4; 4.0
Androstenedione	1.6	1.5	0.7; 2.3
Testosterone	0.7	0.6	0.2; 2.7
Progesterone	2.9	1.6	0.8; 2.9
AEA	1.9	1.7	1.4; 2.4
PEA	6046	4387	3083; 6281
2-AG/1-AG	113	109	75.1; 145
OEA	7006	4312	2492; 6799

4. Conclusion

An LC-MS/MS method that enables the simultaneous quantification

of five steroid hormones and four endocannabinoids in the hair matrix was successfully developed and validated. Special emphasis was placed on an accurate quantification of these endogenous compounds. A combination of surrogate analytes and background correction resulted in a reliable quantification at low pg/mg levels. A first proof-of-concept study with hair samples of 58 individuals showed that cortisone, cortisol, androstenedione, progesterone, testosterone, AEA, PEA, 2-AG and OEA could be detected in the hair matrix. Significant differences in concentrations in female and males were found for testosterone and androstenedione. The results indicated that endocannabinoid concentrations in hair showed strong correlations with each other as well as cortisol with cortisone. The data presented show the potential of the long-term retrospective measurement of endogenous biomarkers in hair. Future studies with larger cohorts could help to define age-, sex-, and color-dependent value ranges for steroids and endocannabinoids.

Credit author statement

Clarissa D. Voegel: Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. Markus R. Baumgartner: Conceptualization, Resources, Writing - Review & Editing, Supervision. Thomas Kraemer: Writing - Review & Editing, Supervision. Stefan Wüst: Resources, Writing - Review & Editing, Funding acquisition. Tina M. Binz: Conceptualization, Resources, Data Curation, Writing - original draft, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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